Alkaline Phosphatase Activity in the Developing Slime Mold, *Dictyostelium discoideum* Raper

by

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
June, 1955

ACKNOWLEDGMENTS

To the members of my graduate committee, Drs. J. H. Gregg, M. Griffith, A. B. Grobman, J. V. Slater and T. W. Stearns, sincere appreciation is expressed for their critical reading of this manuscript.

To Dr. J. H. Gregg, chairman of my graduate committee, I especially express my gratitude for introducing me to Dictyostelium discoideum. Furthermore, it is to him that I am indebted for acquainting me with some of the micro-techniques used in this study.

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INTRODUCTION

During the last decade, the field of enzymology has gained increasing importance as a vehicle with which to gain insight into the general problems of differentiation and morphogenesis. Indications of this are the many reviews and summaries which concern themselves with the relationships of enzymes and embryogenesis (Barth and Barth, 1954; Brachet, 1950; Needham, 1942; Boell, 1948; Gustafson, 1954). A simultaneous rise in our knowledge of the importance of the roles of phosphorus and phosphorylated compounds in metabolic processes has also been evidenced within the last ten years. Their importance is such, "...that the most extraordinary achievement of some future investigator may well be the discovery of a metabolic system in which phosphorus is demonstrably not involved" (Glass, 1952). In the light of these pronounced trends, it is plausible to pursue a problem which attempts to relate the two by embracing a study of an enzyme involved in phosphate metabolism and its possible relationship to the processes of differentiation and morphogenesis. An investigation of alkaline phosphatase activity in the developing slime mold, Dictyostelium discoideum Raper, is such a study.

The enzyme, alkaline phosphatase, has been classified as a hydrolytic enzyme, i.e., its action is one of hydrolysis rather than of synthesis (Roche, 1950). It is activated in the presence of divalent

cations, especially magnesium, and inhibition of activity is accomplished by sulfhydryl compounds and the arsenate ion. The optimal pH for maximum activity is about 9.0-9.5. Substrate specificity is limited to the orthophosphoric monoesters.

The distribution of alkaline phosphatase in the animal kingdom is almost universal. According to Roche (1950), it has been found to exist in every animal cell except those of hyaline cartilage and those of the tunics of vascular vessels, while its presence in the plant kingdom is limited to the bacteria and yeasts.

In terms of possible functional roles of the enzyme, the distribution of alkaline phosphatase within the individual organism has been classified into three main categories (Bradfield, 1950). First, the enzyme is found in sites where active transfer of solutes across cell membranes is known to occur. The kidneys of many mammals, as well as those of other classes of vertebrates, are rich in alkaline phosphatase activity, especially in the region of the proximal convoluted tubule. White and Schmitt (1926) have shown that it is at this level of the kidney that reabsorption of glucose occurs. Blood capillaries (Jacoby, 1946) as well as placentae (Wislocki and Dempsey, 1946) have also been shown to have high degrees of alkaline phosphatase activity.

Secondly, a high correlation has been shown to exist between sites of pronounced alkaline phosphatase activity and areas where calcification processes are occurring. Robison (1923), Fell and Robison (1929), and Moog (1944) have demonstrated this enzyme in the bones of birds and mammals, while Engel and Furuta (1942), and Bevelander and Johnson (1945) have demonstrated the activity of this enzyme in mammalian teeth. The bones, scales, and teeth of fish also exhibit high levels of alkaline phosphatase activity (Roche and Bullinger, 1939). Evidently the enzyme acts in the role of concentrating phosphate ions for the eventual deposition of calcium phosphate (Bradfield, 1950).

In the third instance, alkaline phosphatase has been shown to be intimately associated with areas in which the metabolism of nucleic acids and proteins is occurring. Danielli and Catcheside (1945), and Krugelis (1946) have shown the existence of alkaline phosphatase activity in certain bands of the giant salivary chromosomes of Drosophila. It is in these same bands that desoxyribonucleotides have also been shown to be present. Brachet (1947) has demonstrated a positive correlation between the presence of the enzyme and high DNA turnover in the intestinal mucosa, testis, and spleen of the mouse. In addition, high alkaline phosphatase activity is found in the silk glands of spiders and caterpillars, with the site of activity occurring between the nucleoproteins

of the cytoplasm and the lumen of the gland (Bradfield, 1951). Alkaline phosphatase activity becomes more pronounced with increased differentiation of embryonic tissue when protein synthesis occurs at the expense of yolk (Moog, 1944, 1946; Brachet, 1946; Krugelis, 1947). Bradfield (1946) and Jeener (1947) have expressed the opinion that alkaline phosphatase is concerned with the synthesis of fibrous rather than globular proteins.

With regard to the experimental organism, the life cycle of D. discoideum has been adequately described (Bonner, 1944; Raper, 1935, 1940; Raper and Fennell, 1952). For purposes of orientation, however, a resumé of the life cycle is presented here. Individual myxamoebae (Figure I, 1) germinate from each of the encapsulated spores which are released from the sorogen (spore cap) of the mature sorocarp. The myxamoebae feed upon bacteria and grow in size as well as in numbers. Upon completion of the vegetative stage, aggregation commences and is characterized by a streaming of all myxamoebae toward a central focal point. It is at this focal point that all individual myxamoebae coalesce to form an integrated aggregation mass (Figure I, 2). The streaming phenomenon is thought to be initiated by some chemotactic stimulus (Bonner, 1947). Both syngamy and meiotic activity have been described during the aggregation stage (Wilson, 1953).

As the aggregation mass increases in height, it topples over, and the tip comes in contact with the substrate (Figure I, 3). From this aggregation mass there evolves a migrating pseudoplasmodium (Figure I, 4) which moves over the surface of the substrate. It has been demonstrated that the anterior one-third of the pseudoplasmodium is composed of cells (pre-stalk) which will eventually give rise to the stalk of the mature sorocarp. The remaining portion of the pseudoplasmodium is made up of cells (pre-spore) which will give rise to the spores of the mature individual.

After a period of time determined by environmental conditions (Slifkin and Bonner, 1952), the pseudoplasmodium ceases to migrate and reorganizes itself (Figure I, 5). This individual, which is now in the pre-culmination stage, consists of pre-spore cells with an apex composed of pre-stalk cells. During the succeeding culmination stage (Figure I, 6), the pre-spore mass is raised above the substrate and is supported by the stalk. It is during this developmental stage that the transformation of the individual pre-spore myxamoebae into encapsulated spores takes place. Wilson (1953) has described the occurrence of mitotic activity during the time of spore formation. The raising of the spore mass into the air is continued until the sorogen is finally situated at the highest point of the supporting stalk (Figure I, 7). This is the mature sorocarp.

It has been suggested upon numerous occasions (Bonner, 1944, 1947; Gregg, 1950; Raper and Fennell, 1952) that D. discoideum is ideally suited for the study of differentiation and morphogenesis, since it was thought that in this organism there was no overlap or "masking effect" of growth processes over those of morphogenesis. Wilson's work however seems to cast some doubt upon this concept. Bonner and Frascella (1952) maintain that, even though their observations agree to some extent with those of Wilson, "differentiation is not dependent on mitoses."

It has been the purpose of this work to study the enzyme, alkaline phosphatase, in the slime mold, D. discoideum, from both qualitative and quantitative viewpoints. The qualitative aspect entailed the use of Gomori's method for the histochemical localization of the enzyme (1952) to demonstrate sites of alkaline phosphatase activity in successive stages of the developmental cycle. The second approach was quantitative in that spectrophotometric analyses of alkaline phosphatase activity were made in order to measure the degree of activity in the various stages of development. An attempt is made to correlate the data and observations derived from these methods with already existing biochemical data concerned with differentiation and morphogenesis.

MATERIALS AND METHODS

Culture of Dictyostelium discoideum

D. discoideum was grown in a two-membered culture on a nutrient agar medium. In addition to the spores from the mature sorocarps of D. discoideum, the medium was inoculated with Escherichia coli. The nutrient agar medium was made according to Bonner (1947) and consisted of the following components:

Peptone	10.00 gm.
Dextrose	10.00 gm.
Agar	20.00 gm.
$Na_2HPO_4 \cdot 12H_2O$	0.96 gm.
KH_2PO_4	1.45 gm.
Distilled water to make	1000.00 ml.

Stock cultures were maintained in test tube slants, while cultures from which organisms were harvested for experimentation were grown on media contained in Petri plates.

Histochemical Localization of Alkaline Phosphatase

The method of Gomori (1952) was used to demonstrate histochemically the sites of alkaline phosphatase occurrence. This method is essentially the same as was originally published by Gomori (1939) and Takamatsu (1939). The histochemical localization of alkaline phosphatase is determined by a series of replacement reactions. The tissue is incubated in a buffered medium containing the substrate,

sodium glycerophosphate. The phosphate radical is cleaved and combines with the calcium ion in the medium to form $Ca_3(PO_4)_2$. The tissue is then immersed in a dilute cobalt salt solution, and calcium is replaced by cobalt to form $Co_3(PO_4)_2$. When the tissue is exposed to the final solution, ammonium sulfide, the sulfide replaces the phosphate radical, and black precipitous CoS results. The final precipitate of CoS determines the localization sites of alkaline phosphatase.

Desired stages in the development of D. discoideum were grown as already described. Fixation of the tissue was achieved by flooding the entire Petri plate with ice-cold 80% ethanol. The flooded plate was then put in the refrigerator (5-10° C.) for one hour. After this period of time, the tissues were dehydrated by placing them in ice-cold absolute ethanol for two hours. Cold temperatures were maintained throughout the fixation period so as not to denature the enzyme. After dehydration had been accomplished, the individual organisms were passed through two chloroform baths, approximately fifteen minutes in each bath. From the chloroform, the tissues were passed directly into melted Fisher Tissue-Mat (m.p. 50-52° C.). Oven temperature was maintained at no higher than 55° C., since Gomori (1952) and Danielli (1953) have indicated that sustained high temperatures may destroy the enzymatic activity. When the Tissue-Mat had thoroughly impregnated the tissue (usually one to two hours), the organisms were oriented in the desired position, and the paraffin was allowed to solidify.

All stages of development except the vegetative myxamoebae were processed in this manner. The vegetative myxamoebae were harvested in the manner described by Bonner (1947), and a smear of these cells was made according to Guyer (1953). The smear of myxamoebae was then fixed in 80% ice-cold ethanol and subjected to the histochemical method of Gomori.

All organisms, except the vegetative myxamoebae, were handled individually after the initial fixation with 80% ethanol. Transfers of the tissues from one medium to another were made by means of watchmaker's forceps, hair loops, or the tip of a very fine teasing needle.

All sections were cut at 10 micra. After mounting on slides, the serial sections were incubated in the following medium at 37° C. for three hours:

3% sodium glycerophosphate	10.0 ml.
2% calcium chloride	25.0 ml.
10% magnesium sulfate	10.0 dp.
Sodium barbital	0.7 gm.
Distilled water to make	50.0 ml.

After incubation the slides were immersed in a 2% solution of cobalt chloride for five minutes. Following a two minute rinse in circulating tap water, the serial sections were placed in an ammonium sulfide solution (ten drops of ammonium sulfide in a Coplin jar of distilled

water) for five minutes. To completely wash away all traces of the reagents through which the tissue has been passed, the slides were washed in circulating tap water for at least ten minutes. The sections were then dehydrated and mounted in Piccolyte. Corresponding control slides were made in exactly the same manner with the exception that the substrate, sodium glycerophosphate, was omitted from the incubating medium.

Spectrophotometric Analysis of Alkaline Phosphatase Activity

Spectrophotometric analyses of alkaline phosphatase activity were performed in essentially the same manner as was done by Krugelis, et al. (1952) and Krugelis (1950). Four stages in the developmental cycle of D. discoideum were chosen on which to do the analyses. These were the vegetative myxamoebae, migrating pseudoplasmodia, young sorocarps, and mature sorocarps. Harvesting of myxamoebae was done in the manner already described. The individual migrating pseudoplasmodia and young sorocarps were picked from the agar surface with the aid of a hair loop and transferred to the grinding surface of the homogenizer. The young sorocarp stage was defined as that stage of development in which the sorogen had been raised above the substrate but in which the individual was not as yet mature. Watchmaker's forceps were used to harvest the mature sorocarps individually.

In view of the fact that the pseudoplasmodium crawls over the same substrate upon which E. coli is feeding, several experiments were performed (Gregg, 1954) to determine whether E. coli was being harvested with the slime mold and contributing any enzymatic activity to that in the slime mold. D. discoideum was allowed to develop to the point where aggregation masses were formed. Circular discs of agar upon which the aggregation masses were situated were then cut out with a large size cork borer. These discs were transferred to cut-outs of exactly the same size which were previously made in non-nutrient agar medium. The aggregation masses then developed into migrating pseudoplasmodia which migrated off the nutrient agar discs onto the non-nutrient agar substrate. It was believed that any E. coli which might have adhered to the pseudoplasmodium as it migrated from the nutrient agar would be lost as it migrated over the non-nutrient agar. Because no pronounced differences in final results were evident between experiments performed on individuals gathered from nutrient and non-nutrient agar, the former procedure was continued.

Organisms, harvested in the desired stage of development, were homogenized in a micro-homogenizer of the type described by Gregg, et al. (1954). Water was used as the extracting medium. The homogenate was diluted to a volume of 1.0 ml. and thoroughly mixed. With

the use of micro-pipettes, aliquots of the homogenate were taken to determine tissue dry weight as well as alkaline phosphatase activity.

Tissue dry weight determinations were made in the following manner: An aliquot of the homogenate was taken and transferred to a thin circular collodion membrane having the diameter of a No. 3 cork borer. Since the size of the membrane could not accomodate more than 20 µl of brei at one time, several 20 µl fractions were added on the membrane, with each fraction being dried before the succeeding fraction was added. After a sufficient amount of the homogenate had been added, the preparations were placed in a drying oven at 60° C. for a period of twelve hours. The tissue was weighed on a quartz helical balance having a range of from 1.0 to 1000 µg.

Two 0.3 ml. aliquots of the tissue brei were used for measuring alkaline phosphatase activity, and one 0.3 ml. aliquot was used as a corresponding control. The three aliquots were centrifuged to remove all cell debris and particulate matter. The supernatants, which served as the source of enzyme, were quantitatively transferred to three separate tubes. To each of these three tubes an equal volume of buffered substrate was added and then thoroughly mixed. The buffered substrate was composed of the following components:

Sodium glycerophosphate	10 mg/ml H_2O
Sodium barbital	40 mg/ml H_2O
Magnesium sulfate	2.5 mg/ml H_2O

The pH of the buffered substrate, 9.3, was measured by means of a Beckman pH meter.

Immediately upon the addition of the buffered substrate to the enzyme extract, the reaction in the control tube was stopped with the addition of an equal amount (0.6 ml) of 25% trichloroacetic acid (TCA). The experimental tubes, however, were allowed to incubate at room temperature for a period of three hours, and then the enzymatic reaction was also stopped with an equal amount of 25% TCA. Following this, the three tubes, two experimentals and one control, were centrifuged to remove the precipitate.

The supernatants were quantitatively transferred to a color reaction vessel, and a measure of the amount of phosphorus liberated from the substrate by alkaline phosphatase (a measure of alkaline phosphatase activity) was made colorimetrically. The determination was done according to the procedure of Kuttner and Cohen (1937), with modifications suggested by Krugelis (1950). This method is based on the reduction of phosphomolybdic acid by stannous chloride with a subsequent reading of the intensity of the formed color in a Beckman DU Spectrophotometer at 700 μ.

To each of the supernatants was added each of the following reagents in the order listed:

4 N sulfuric acid	1.0 ml.
3% ammonium molybdate	1.0 ml.
0.06% stannous chloride	1.0 ml.

After the addition of the last reagent, a period of fifteen minutes was allowed for the color to develop. The color intensity was then read.

The amounts of phosphorus liberated were determined by comparing experimental extinction values with the extinction values of standard solutions containing known amounts of phosphorus. Results are expressed in terms of P liberated during three hours of incubation at 25° C. per µg dry weight of tissue.

Several preliminary experiments were performed to determine the optimal pH, temperature, and substrate concentration. These experiments indicated that pH 9.3, 25° C. incubating temperature, and a substrate concentration of 10 mg/ml H_2O were most favorable for optimal enzymatic activity.

RESULTS

Histochemical Localization of Alkaline Phosphatase

The results of the histochemical localization of alkaline phosphatase appear in Figures II through XII. The photomicrograph of the vegetative myxamoebae (Figure IIa) has been supplemented with a drawing (Figure IIb) made from a region of the same preparation in order to present more clearly the sites of enzymatic activity. Figures III through XII represent sections of individual slime molds presented in successive stages of development. Control slides were made simultaneously, but in no case was any darkening of the tissue obtained. Therefore, all dark areas on the experimental sections have been considered sites of enzymatic activity. Photomicrographs of control slides are not presented because lack of contrast prevented the taking of clear pictures.

Figures IIa and IIb.

In the myxamoebae, sites of alkaline phosphatase activity can be seen in the cytoplasm immediately adjacent to the nuclear membrane. It is reasonably certain that no connection exists between these sites of activity, which occur several to a cell. Other sites of activity are noted in the cytoplasm, but they are not as pronounced in size as those next to the nuclear membrane.

Figure III.

This is a section of a pseudoplasmodium evolving from an aggregation mass. An area of pronounced alkaline phosphatase activity is evident in the most anterior extreme of the organism. The cells of the aggregation mass are compact toward the periphery, while the stream of cells forming the pseudoplasmodium exhibits a loose texture. The enzymatic activity in the cells of the entire organism, except at the most anterior end, is comparable to the staining seen in the vegetative amoebae.

Figure IV.

This section represents a relatively young migrating pseudoplasmodium. The cells of the anterior end, i.e., the pointed end, are beginning to align themselves in a plane perpendicular to the longitudinal axis of the organism. This orientation becomes more evident in the late migrating pseudoplasmodium. Not all young migrating pseudoplasmodia, however, exhibit this cell orientation. This is in agreement with the findings of Bonner (1944). The anterior region also exhibits more activity as compared to the remainder of the section. This is the region of pre-stalk cells.

Figure V.

This is a section of a late migrating pseudoplasmodium which will shortly cease migrating and begin to culminate. The cells in

the anterior one-third of the section (pre-stalk region) are definitely aligned in a plane perpendicular to the longitudinal axis of the organism. The cells in the posterior two-thirds of the organism (pre-spore region) are oriented randomly. A difference in the staining characteristics of the two regions is also evident, the pre-stalk region being slightly darker than the pre-spore region.

Figure VI.

In the stage of development represented in this photomicrograph, the slime mold has ceased migrating and the antero-posterior axis assumes a vertical orientation with respect to the substrate. A configuration of cells resembling a crescent is seen in the apical portion of the section. In the entire organism, these cells form an open-ended cylinder, the sides of which appear as two vertical lines of cells in the photograph. These form the sides of the crescent which on close examination can be seen to lack a connection across the top. The walls of the cylinder are one cell wide. These cells are horizontally aligned and lay one atop the other. The level at which this cylinder occurs strongly suggests that it is concerned with the formation of the future sorophore sheath which will enclose the stalk of the organism.

The cells inside the cylinder are beginning to show vacuolization. They are round in shape and exhibit alkaline phosphatase activity to a lesser degree than the cells of the cylinder surrounding them. The

localization of the enzyme in these cells is not as diffuse as in the cylinder cells but rather appears to be limited to definite "spots" of activity. The semi-vacuolated state of these cells indicates that they are in the process of transforming into stalk cells.

The cells outside the cylinder have also become elongated, approaching the shape of the cylinder cells. They have assumed a horizontal orientation rather than the vertical orientation of the pre-stalk cells of the migrating pseudoplasmodium. Their location in the organism suggests that they are components of the pre-stalk region. The staining of these cells is lighter than that found in the cylinder cells.

At the uppermost extreme of the section, the horizontally aligned cells above the cylinder merge imperceptibly with the rounded cells already described. These latter in turn enter the area between the walls of the cylinder and grade into the cell mass which is undergoing vacuolization.

The remainder of the section is composed of the pre-spore mass. In this area there is no evidence of any high degree of alkaline phosphatase activity taking place. Activity appears to be at a minimum. The cells of this region show no elongation but

retain their rounded configuration. Also, there is no continuation of the cylinder in this part of the organism, and in no portion of the slime mold has the sorophore sheath appeared as yet.

Figure VII.

This is a section of an individual more advanced in development than that in Figure VI. The sorophore sheath, i.e., the membrane which will enclose the stalk proper, is especially well defined. Its length, however, is limited to the region visible in the photograph. The vacuolated cells, which for the most part are surrounded by the sheath, extend below the lower limits of the sheath into the pre-spore mass, but as yet they do not reach to the substrate.

Certain characteristics, indications of which could be seen in Figure VI, have now become more obvious. The horizontal elongation of the cells outside the sorophore sheath and the pronounced alkaline phosphatase activity in these cells is easily seen. Evident also is the lack of continuity across the upper end of the sorophore sheath. It appears that the cells at the apex of the section flow over the upper edges of the sheath into the area of the stalk itself. These are round cells which show only a slight degree of alkaline phosphatase activity. Progressing down the stalk region, increased

vacuolization of the stalk cells is evident. At the lowest limit of the stalk, this process is complete. Enzymatic activity is not evident in the completely vacuolated cells. In the surrounding pre-spore mass at the base of the section, alkaline phosphatase activity is at a minimum.

Figure VIII.

In this stage of development, the pre-spore mass has begun to rise above the substrate. A band of heavy staining is seen in the center of the section which completely encircles the organism at this level. Its position suggests that it is the portion of the pre-stalk region immediately adjacent to the upper limits of the pre-spore region. A very weak positive staining is evident in the pre-spore region. Above the band of heavy staining can again be seen the horizontally aligned cells which, as before, merge with the rounded cells at the apex of the section. Here, as in Figure VII, continuity of the sorophore sheath at its upper limits can not be observed.

Examination of all the serial sections of this individual reveals that the stalk as well as the sorophore sheath now extend through the center of the organism to the substrate. A gradient of increasing vacuolization in the stalk cells is apparent starting from the rounded cells of the upper extreme to the completely

vacuolated cells in contact with the substrate. A corresponding decrease in staining parallels this gradient.

Figure IX.

In this section the pre-spore mass has risen almost clear of the substrate. The entire length of stalk is visible and reveals the transition of the stalk cells from the semi-vacuolated type at the top of the stalk to the completely vacuolated cells at the bottom. Portions of the sorophore sheath can be seen in the upper and lower halves of the stalk. Here, as in Figures VII and VIII, the horizontally aligned cells surround the upper portion of the stalk.

A new trend exhibits itself in this section. An exceedingly dark-staining area is apparent in the lower half of the pre-stalk region. It is much darker than any positive staining reaction seen thus far in the developmental cycle. The darkness of the staining suggests that a high degree of alkaline phosphatase activity occurs in this region. This highly active area is sharply demarcated from the lower pre-spore mass. The staining extends completely around the organism at this level and is separated from the stalk area by the sorophore sheath.

No sites of enzymatic activity were evident in the completely vacuolated cells of the lower stalk. The cells of the upper stalk,

however, being incompletely vacuolated, showed a slight degree of activity. A similar low degree of activity was found in the pre-spore mass.

Figure X.

This is a section through a culminating individual with the pre-spore mass raised completely above the substrate. Many of the same features can be pointed out in this section as were present in Figure IX. These are the high degree of enzymatic activity in the horizontally aligned cells of the pre-stalk region, the weakly active, semi-vacuolated cells of the upper stalk, the completely vacuolated lower stalk cells showing no activity, and the presence of the sorophore sheath.

In addition, several new features are present for the first time. The cells of the pre-spore mass have begun to transform into mature spore cells. It is during culmination that the pre-spore myxamoebae undergo this transformation (Bonner, 1944; Raper and Fennell, 1952). Isolated areas of enzymatic activity are seen in the sorogen. Furthermore, a second area of intense staining is found for the first time in the lower region of the sorogen. It extends downward, in close association with the stalk, into the basal disc region. The intensity of staining in this region is very much like that found in the pre-stalk area. Another curious similarity is the fact that here, too, the cells are aligned horizontally and elongated.

Figure XI.

In this individual, development has progressed further than in Figure X with the sorogen being raised appreciably higher above the substrate. The expanse and intensity of staining is quite striking. The pre-stalk region maintains its intense staining as before. An extension of this stained area however is seen to reach down into the spore mass and meets a similar upward extension from the darkly stained area at the base of the spore mass. This connecting portion is confined to approximately the width which is seen in the photomicrograph and does not extend out to the periphery of the spore mass. The area of intense staining at the base of the spore mass runs downward for some distance along the stalk. The complete absence of any alkaline phosphatase activity in the stalk cells is again noted.

Figure XII.

This is a section through the spore cap of a mature individual. A portion of the stalk is seen imbedded in the spore mass. Actually, at this stage the stalk extends from the substrate to the uppermost tip of the spore cap. A minimum of activity is evident in the spore cells, while no activity was observed in the stalk cells.

Spectrophotometric Analysis of Alkaline Phosphatase Activity

The data derived from the spectrophotometric analyses of alkaline phosphatase activity are found in Table I and summarized in Table II.

Table I

Myxamoebae	Migrating Pseudoplasmodia	Young Sorocarps	Mature Sorocarps
0.493	0.667	1.57	0.191
0.505	0.667	1.52	0.219
0.628	0.581	1.47	0.150
0.598	0.545	1.45	0.210
0.568	0.626	1.11	0.115
0.538	0.626	1.15	0.092
0.629	0.540		0.117
0.629	0.602		0.163
	0.578		0.114
			0.137
			0.102
			0.153

Individual experimental data of spectrophotometric analyses of alkaline phosphatase activity. Results are expressed in terms of μg P liberated/μg tissue dry weight. These data represent the actual values x 10^2.

Figure XIII illustrates the results graphically. From these data it may be stated that:

1. When the enzymatic activity in the migrating pseudoplasmodia is compared to that in the vegetative myxamoebae, no statistical difference is evident ($P > 0.05$)[1].

2. A 113% increase in alkaline phosphatase activity is exhibited by the young sorocarps relative to the migrating pseudoplasmodia and the vegetative myxamoebae. This difference is shown to be statistically significant ($P < 0.001$).

3. Following this increase in activity in the young sorocarps over the migrating pseudoplasmodia and vegetative myxamoebae, an abrupt and pronounced decrease in activity is found in the mature sorocarps as compared to the young sorocarps ($P < 0.001$).

4. Analysis of enzymatic activity in the myxamoebae indicates that an increase in activity occurred relative to the mature sorocarps ($P < 0.001$).

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[1] Student's "T" test was used to calculated probability values.

TABLE II

Stage of Development	No. of Experiments	µg P/µg dry wt. Mean ± S.D.
Myxamoebae	8	0.574 ± 0.053
Migrating Pseudoplasmodia	10	0.597 ± 0.046
Young sorocarps	6	1.380 ± 0.180
Mature sorocarps	12	0.147 ± 0.040

Summary of data in Table I. Mean values of µg P liberated/ µg dry tissue weight with their standard deviations. These data represent the actual values x 10^2.

DISCUSSION

Both histochemical and spectrophotometric techniques were used to study alkaline phosphatase activity in D. discoideum. The information gained by one method was complemented by the results shown by the other. The spectrophotometric analyses showed no difference in activity between the migrating pseudoplasmodia and the vegetative myxamoebae. However, a two-fold increase in activity occurred in the young sorocarps relative to both the myxamoebae and migrating pseudoplasmodia. In the mature sorocarps, the activity dropped to a minimum.

Based on the fact that the histochemical approach can be quantified to a certain degree (Gomori, 1952; Danielli, 1953), the same conclusions can be derived from the histochemical approach to this problem. During the course of development, only slight differences in intensity of staining were evident in the stages represented in Figures II through VIII. It is interesting to note that these differences, although slight, denoted increased activity only in the pre-stalk region. In Figure IX, a young sorocarp, an area of extremely dark staining became evident in the pre-stalk region. Thus, alkaline phosphatase activity increased in this region as compared to the activity in the pre-stalk area in the preceding stage. In succeeding stages, the activity in the same region not only maintained its intense staining characteristic but became more

widespread, as is seen in Figures X and XI. Furthermore, enzymatic activity was observed in the region of the sorogen. The activity declined rapidly in the mature sorocarp and dropped to a minimum.

Not only do the results of these two methods of approach complement each other, but they are as well correlated with other works done on D. discoideum. Bonner (1944) has shown that transformation of the pre-spore cells into mature spore cells first occurs at the upper periphery of the sorogen. Transformation then proceeds rapidly inward and downward through the sorogen untill all pre-spore cells are differentiated. An inspection of Figure XI which represents an individual approaching maturity, indicates that a somewhat similar pattern can be seen in the alkaline phosphatase localization. It is doubtful that this is a mere coincidence. The evidence would suggest that alkaline phosphatase activity is intimately concerned with the transformation of pre-spore cells to mature spore cells. The occurrence of alkaline phosphatase activity in both the pre-stalk area and sorogen of the young sorocarp is reflected in the spectrophotometric data by a 113% increase in activity over that found in the migrating pseudoplasmodia where somewhat pronounced activity, as revealed by the histochemical technique, was found only in the pre-stalk area.

In Figure X, alkaline phosphatase activity was demonstrated at the lower periphery of the sorogen as well as in the pre-stalk region. The cells in this region of the sorogen were aligned in nearly a transverse direction. Raper and Fennell (1952) also found such an orientation of the cells in this region in the same stage of development. Regarding this region, these authors believe that the cells therein "....exert an appreciable but continually decreasing role in raising the sorogen during culmination." The coincidence of such a postulate for the function of this region and the occurrence of high alkaline phosphatase activity in the same region would suggest that this enzyme may be concerned in some manner with this process.

Histochemical analyses have shown that alkaline phosphatase activity is most pronounced in the pre-stalk region. The pronounced activity in the pre-stalk region first occurred in the young migrating pseudoplasmodium and persisted during development until shortly before the formation of the mature sorocarp. In view of the fact that the pre-stalk region gives rise to the stalk, it would seem reasonable to suggest that alkaline phosphatase is concerned in some manner with certain mechanisms involved in the formation of the stalk. Other observations seem to substantiate this view. It was pointed out that in the culminating individual, the cells of the pre-stalk area assume

a horizontal or transverse orientation in addition to the fact that they become very densely stained. As these cells are followed upward over the open end of the sorophore sheath and into the sorophore proper, the horizontal cells merge with rounded, randomly oriented cells which are becoming vacuolated. These cells are then seen to enter the inside of the sorophore sheath and become completely vacuolated, cellulose-containing cells. A change in the enzyme staining characteristics is directly associated with this gradual change in cell architecture. In the horizontally aligned cells, a dark staining reaction occurs which decreases in intensity in the semi-vacuolated, round cells. A cessation of activity was found in the completely vacuolated cells of the stalk proper.

By the use of numerous qualitative tests, Raper and Fennell (1952) were able to demonstrate quite conclusively that the main component of the sorophore was cellulose. According to them, the cellulose of the stalk cells, as well as the spore cells, is deposited intracellularly. They further state that the first cellulose formed is deposited as a "....continuous film (sorophore sheath) adjacent to the surface of a layer or palisade of cells actively secreting cellulose synthesizing enzymes." An inspection of Figure III reveals not only two such palisades (the sides of the

described cylinder) but also reveals that the cells of these palisades are elongated horizontally and are more pronounced in alkaline phosphatase activity than are the cells on either side of them. On the basis of relationships between these studies, it is tempting to suggest that alkaline phosphatase is concerned with the formation of the sorophore sheath.

Gregg et al. (1954) and Gregg and Bronsweig (1954) found an inverse relationship existing between protein content and amount of reducing substance (presumably carbohydrate). This suggested that the synthesis of cellulose in the slime mold occurs at the expense of protein. As the amount of protein decreases during development, the quantity of reducing substance increases. In addition, alkaline phosphatase has been demonstrated in the present work in regions where cellulose is being formed. In the light of these facts, the following postulate is presented in an attempt to explain the manner in which protein is converted to cellulose. The limits of this postulate are defined not only by the comparative lack of biochemical data concerned with D. discoideum, but also by the lack of more specific knowledge concerning the relationship of alkaline phosphatase to other enzymes and enzyme systems.

It was shown by Gregg, et. al. (1954) that the metabolism of proteinaceous substances increased as the development of the slime

mold progressed. If this protein, or part of it, exists in the form of a nucleoprotein complex, then, presumably alkaline phosphatase is instrumental at this point in liberating the protein from the nucleic acid as was suggested by Bradfield (1951). This is not to say that alkaline phosphatase per se is the enzyme exercising such a function. In all likelihood, it would be part of an enzyme system acting in this capacity as was also suggested by Bradfield. The released protein could then enter an enzyme system for its conversion to carbohydrate. In view of the current concept involving phosphorylase and phosphatase actions for the biosynthesis of polysaccharides (Lipmann, 1941), it is conceivable that this released protein after conversion to carbohydrate could be intracellularly deposited as the final product, cellulose, in the spore cells and stalk cells. In view of the "versatility" of action of alkaline phosphatase, it is further conceivable that the enzyme may act again in this conversion by virtue of its phosphatase action. Pronounced alkaline phosphatase activity was noted during that period of development when stalk cells were being formed, as well as in that area of the young sorocarp where the transformation of pre-spore to mature spore cells containing a cellulose covering was occurring.

As was indicated earlier, the sorophore sheath is thought to be formed next to cells secreting cellulose synthesizing enzymes (Raper

and Fennell, 1952). Moog and Wenger (1952) demonstrated the occurrence of high alkaline phosphatase activity and high concentrations of mucopolysaccharides in close association with each other. These authors went so far as to suggest that alkaline phosphatase itself was the polysaccharide moiety of the mucopolysaccharide complex. If so, the added evidence that alkaline phosphatase plays a "....part in the carriage of organic substances across membrane barriers" (Yao, 1950), strongly suggests the possibility that alkaline phosphatase is also concerned with the extracellular deposition of cellulose to form the sorophore sheath. Whether it acts the role of enzyme in this function or whether it serves as the source of the polysaccharide is as yet not known.

An attempt has been made to establish a series of correlations between the data and observations of this work with previously known facts concerning the biochemistry of Dictyostelium. With additional work on this organism, now contemplated, it is felt that alkaline phosphatase will prove to play an important role in the developmental processes in Dictyostelium discoideum as was suggested here.

SUMMARY

1. Techniques pertinent to the histochemical and spectrophotometric analysis of alkaline phosphatase activity in the developing slime mold, Dictyostelium discoideum, are described.

2. The results of the histochemical techniques as applied to different stages in the development of the slime mold showed the following: sites of enzymatic activity in the vegetative myxamoebae were evidenced next to the nuclear membrane as well as in the cytoplasm itself.

Enzyme activity in the migrating pseudoplasmodium, as well as in the preculmination stage, is limited for the most part to the pre-stalk area. This activity is not too pronounced as compared to that which is demonstrated in the young sorocarp. In the young sorocarp, increased activity can be seen in the pre-stalk regions of the individual slime molds. This increased activity is expressed in terms of not only darker staining characteristics but also in terms of a more wide spread distribution of the activity than was found in the previous two stages.

3. The quantitative spectrophotometric analyses show that no appreciable change in activity occurs in the myxamoebae and the

migrating pseudoplasmodia, relative to each other. A 113% increase in activity is measured, however, in the young sorocarp stage relative to the previous two stages of development. An analysis of enzyme activity in the mature sorocarps reveals that the activity drops to a level below that found in the myxamoebae.

4. The results of this work are discussed in the light of other studies on Dictyostelium discoideum in an attempt to describe the role which alkaline phosphatase may have in the development of this organism.

ILLUSTRATIONS

Figures III through XII are photomicrographs of serial sections of the slime mold, Dictyostelium discoideum. Each of the sections is cut at 10 μ, and they are presented in successive stages of development. Below each photomicrograph is a scale denoting 150 μ.

The vegetative myxamoebae are shown in Figure II; (a) a photomicrograph, (b) a pen drawing. The myxamoebae average 5 μ in diameter.

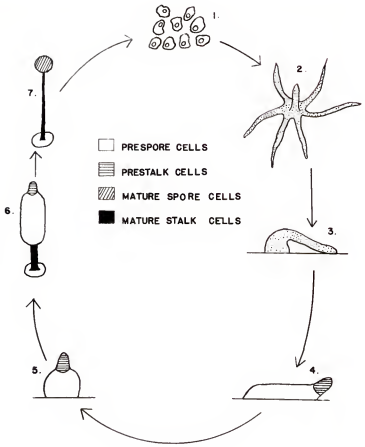

Figure I

Life cycle of the slime mold, *Dictyostelium discoideum*

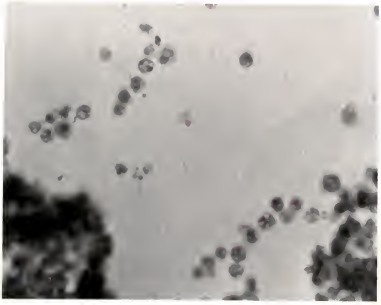

Figure IIa

Photomicrograph of vegetative myxamoebae

Figure IIb

Drawing of vegetative myxamoebae showing sites of alkaline phosphatase not too clearly defined in Figure IIa

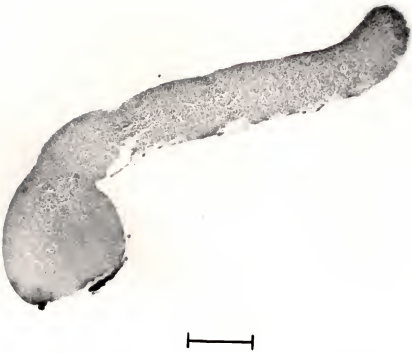

Figure III

The evolution of a pseudoplasmodium
from an aggregation mass

Figure IV

An early or "young" pseudoplasmodium

Figure V

A late or "old" pseudoplasmodium

Figure VI

A pseudoplasmodium which has ceased migrating and has righted itself

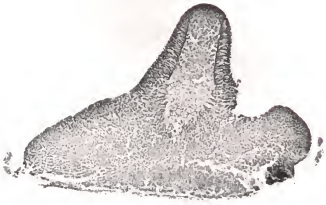

Figure VII

An individual in the preculmination stage

Figure VIII

Mid-preculmination stage succeeding
the previous figure

Figure IX

An individual in which the serogen has been almost raised clear of the substrate

Figure X

Young sorocarp in the culmination stage

Figure XI

A nearly mature sorocarp in late culmination stage

Figure XII

A mature sorocarp

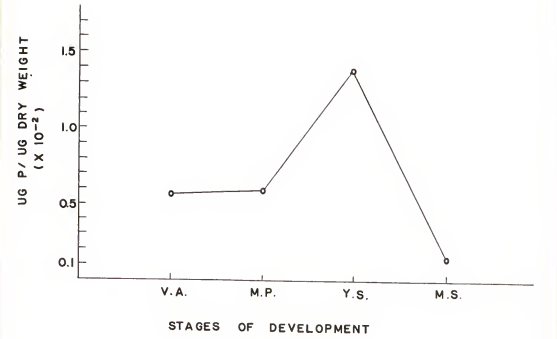

Figure XIII

Spectrophotometric analysis of alkaline phosphatase activity. Graphic presentation of data in Table II. V.A. (vegetative amoebae); M.P. (migrating pseudoplasmodia); Y.S. (young sorocarps); and M.S. (mature sorocarps). These values are to be multiplied by 10^{-2}.

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BIOGRAPHICAL ITEMS

Jerome Oldrich Krivanek was born December 27, 1924, at Chicago, Illinois. He attended elementary school and high school in that city and graduated from Farragut High School in 1941. He attended Herzl Junior College, Chicago, Illinois, from 1941 to 1943.

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He attended the Graduate School of the same university and received the Master of Science degree in physiological zoology in February, 1951. In September of that year, he enrolled in the Graduate School of the University of Florida and has been engaged in studies and research leading to the degree of Doctor of Philosophy since that date. He has served as a research assistant for the academic year, 1952-1953, and as a graduate assistant for the remainder of the time.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 6, 1955

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